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Thiolation of Protein-bound Carcinogenic Aldehyde

AN ELECTROPHILIC ACROLEIN-LYSINE ADDUCT THAT COVALENTLY BINDS TO THIOLS*

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Acrolein, a representative carcinogenic aldehyde that could be ubiquitously generated in biological systems under oxidative stress, shows facile reactivity with the ε-amino group of lysine to form N^{ε}-(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine) as the major product (Uchida, K., Kanematsu, M., Morimitsu, Y., Osawa, T., Noguchi, N., and Niki, E. (1998) *J. Biol. Chem.* 273, 16058–16066). In the present study, we determined the electrophilic potential of FDP-lysine and established a novel mechanism of protein thiolation in which the FDP-lysine generated in the acrolein-modified protein reacts with sulfhydryl groups to form thioether adducts. When a sulfhydryl enzyme, glyceraldehyde-3-phosphate dehydrogenase, was incubated with acrolein-modified bovine serum albumin in sodium phosphate buffer (pH 7.2) at 37 °C, a significant loss of sulfhydryl groups, which was accompanied by the loss of enzyme activity and the formation of high molecular mass protein species (>200 kDa), was observed. The FDP-lysine adduct generated in the acrolein-modified protein was suggested to represent a thiol-reactive electrophile based on the following observations. (i) N^{α}-acetyl-FDP-lysine, prepared from the reaction of N^{ε}-acetyl lysine with acrolein, was covalently bound to glyceraldehyde-3-phosphate dehydrogenase. (ii) The FDP-lysine derivative reacted with glutathione to form a GSH conjugate. (iii) The acrolein-modified bovine serum albumin significantly reacted with GSH to form a glutathiolated protein. Furthermore, the observation that the glutathiolated acrolein-modified protein showed decreased immunoreactivity with an anti-FDP-lysine monoclonal antibody suggested that the FDP-lysine residues in the acrolein-modified protein served as the binding site of GSH. These data suggest that thiolation of the protein-bound acrolein may be involved in redox alteration under oxidative stress, whereby oxidative stress generates the increased production of acrolein and its protein adducts that further potentiate oxidative stress via the depletion of GSH in the cells.

Several lines of evidence indicate that the oxidative modification of protein and the subsequent accumulation of the modified proteins have been found in cells during aging and oxidative stress and in various pathological states including premature diseases, muscular dystrophy, rheumatoid arthritis, and atherosclerosis (1–4). The important agents that give rise to the modification of a protein may be represented by reactive aldehydic intermediates such as 2-alkenals and 4-hydroxy-2-alkenals (3, 5, 6). These reactive aldehydes are considered important mediators of cell damage because of their ability to covalently modify biomolecules, which can disrupt important cellular functions and cause mutations (5). 2-Alkenals represent a group of highly reactive aldehydes containing two electrophilic reaction centers. A partially positive carbon 1 or 3 in such molecules can attack a nucleophile such as a protein. Among all the α,β-unsaturated aldehydes, acrolein is by far the strongest electrophile (5). Acrolein is widely found in the environment and is formed in cells via lipid peroxidation (7). Its high reactivity indeed makes this aldehyde a dangerous substance for the living cell. A number of reports have appeared describing the damaging effects of acrolein on the tracheal ciliatory movement (8) and the pulmonary wall (9). It has also been shown that acrolein reduces the colony-forming efficiency of mammalian cells, forms cyclic adducts with nucleosides *in vitro*, and is a potent mutagen (5). Moreover, acrolein was shown to initiate urinary bladder carcinogenesis in rats (10).

We have investigated the reaction of protein with acrolein and identified a novel acrolein-lysine adduct, N^{ε}-(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine),[1] as the major product (7). In addition, by using a monoclonal antibody (mAb5F6) against FDP-lysine, we have shown that FDP-lysine recognized by the antibody indeed constitutes the atherosclerotic lesions in which intense positivity is primarily associated with macrophage-derived foam cells (11). These findings and the *in vitro* observations (7) that (i) FDP-lysine was detected in the oxidatively modified low-density lipoprotein with Cu^{2+} and (ii) a metal-catalyzed oxidation of arachidonate was associated with the formation of acrolein suggest that polyunsaturated fatty acids may represent the potential sources of acrolein generated during the peroxidation of low density lipoprotein (LDL). In the present study, we found that FDP-lysine is not the end product but is the electrophilic intermediate that potently reacts with thiol compounds. Moreover, we established a novel mechanism of protein thiolation in which FDP-lysine generated in the acrolein-modified protein undergoes the nucleophilic addition of thiols. In addition to the mechanisms for protein *S*-thiolation, our present findings suggest an alternative mechanism that utilizes the acrolein-lysine adduct (FDP-lysine) as a potential thiol-binding site.

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[1] The abbreviations used are: FDP-lysine, N^{ε}-(3-formyl-3,4-dehydropiperidino)lysine; LDL, low density lipoprotein; BSA, bovine serum albumin; LC-MS, liquid chromatography-mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption and ionization time-of-flight mass spectrometry; HPLC, high performance liquid chromatography; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; Tris-buffered saline; DNPH, 2,4-dinitrophenylhydrazine; HIV, human immunodeficiency virus.

EXPERIMENTAL PROCEDURES

Materials—N^{α}-acetyl-L-lysine, acrolein, GSH, N^{α}-acetylcysteine, glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), and bovine serum albumin (BSA) were obtained from Sigma. The horseradish peroxidase-linked anti-rabbit IgG immunoglobulin and ECL (enhanced chemiluminescence) Western blotting detection reagents were obtained from Amersham Biosciences.

General Procedures—Liquid chromatography-mass spectrometry (LC-MS) was measured with a Jasco PlatformII-LC instrument. Matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed using a Voyager-DE PRO mass spectrometer (Applied Biosystems, Tokyo, Japan) operated in the linear mode.

Preparation of N^{α}-Acetyl-FDP-lysine—N^{α}-Acetyl-FDP-lysine was prepared by incubating 100 mM N^{α}-acetyl lysine with 100 mM acrolein in 50 mM sodium phosphate buffer (pH 7.2) for 24 h at 37 °C. N^{α}-acetyl-FDP-lysine was purified by a reverse-phase HPLC using a Develosil ODS-HG-5 column (4.6 × 250 mm) (Nomura Chemicals, Seto, Japan) equilibrated in a solution of 5% methanol in 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min. The elution profiles were monitored by absorbance at 227 nm. The chemical structure of N^{α}-acetyl-FDP-lysine was confirmed by the LC-MS analysis and ^{1}H- and ^{13}C-NMR spectrometries (7).

Preparation of Acrolein-modified BSA—BSA (1.0 mg/ml) was incubated with 10 mM acrolein in 50 mM sodium phosphate buffer (pH 7.2) at 37 °C for 24 h. The reaction mixture was then applied to a PD-10 column (Sephadex G-25) equilibrated with 0.1 M sodium phosphate buffer (pH 7.2) to separate the acrolein-modified protein from the free acrolein. The amount of FDP-lysine generated in the acrolein-modified BSA was examined by amino acid analysis as previously reported (7).

Inactivation of GAPDH by Acrolein-modified BSA or N^{α}-Acetyl-FDP-lysine—Rabbit muscle GAPDH (0.1 mg/ml) was incubated with acrolein-modified BSA (0–0.1 mg/ml) or N^{α}-acetyl-FDP-lysine (0–1 mM) in 1 ml of 50 mM sodium phosphate buffer (pH 7.2) for 2 h at 37 °C. The enzyme activity of GAPDH was measured as previously reported (12). In brief, a 10-μl aliquot of the reaction mixture was assayed in 3 ml of 15 mM sodium pyrophosphate containing 30 mM sodium arsenate buffer (pH 8.5). The reaction of GAPDH was initiated by the addition of 100 μl of 7.5 mM NAD, 100 μl of 0.1 mM dithiothreitol, and 100 μl of 15 mM DL-glyceraldehyde-3-phosphate. The mixture was incubated at room temperature for 5 min, and the absorbance at 340 nm was measured. The amount of the sulfhydryl group was fluorometrically measured according to the method of Hissin and Hilf (36). In brief, 1.8 ml of 0.1 M phosphate solution (pH 8.0) containing 5 mM EDTA and 100 μl of the *o*-phthalaldehyde solution (1 mg/ml) were added to the reaction mixture (100 μl), and then the fluorescence intensity at 420 nm was determined with activation at 350 nm.

Reaction of N^{α}-Acetyl-FDP-lysine with N^{α}-Acetylcysteine or GSH—N^{α}-Acetyl-FDP-lysine (1 mM) was incubated with N^{α}-acetylcysteine (10 mM) or GSH (10 mM) in 50 mM sodium phosphate buffer (pH 7.2) at 37 °C. The reaction products of N^{α}-acetyl-FDP-lysine with GSH were examined by reverse-phase HPLC using a Develosil ODS-HG-5 column (4.6 × 250 mm) (Nomura Chemicals) equilibrated in a solution of 5% methanol in 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min. The elution profiles were monitored by absorbance at 227 nm.

Reaction of Acrolein-modified BSA with GSH—The acrolein-modified BSA (1.0 mg/ml) was incubated with 10 mM GSH in 50 mM sodium phosphate buffer (pH 7.2) at 37 °C.

Determination of FDP-lysine Contents in the Acrolein-modified BSA by Amino Acid Analysis—An aliquot (0.1 ml) of the protein samples was treated with 10% trichloroacetic acid. After centrifugation at 10,000 × *g* for 3 min, the proteins were hydrolyzed *in vacuo* with 6 N HCl for 24 h at 105 °C. The hydrolysates were then concentrated and dissolved in 50 mM sodium phosphate buffer (pH 7.4). The amino acid analysis was performed using a JEOL JLC-500 amino acid analyzer equipped with a JEOL LC30-DK20 data analyzing system.

Acrolein-trapping Enzyme-linked Immunosorbent Assay (ELISA)—To coat the wells of the microtiter plate, 100 μl/well poly-L-lysine (1 mM, lysine equivalent) in 50 mM sodium phosphate buffer (pH 7.2) was used and then incubated overnight at 4 °C. Following washing with TBS containing 10% Tween 20 (TBS/Tween), 100 μl of the acrolein solution (10 mM) was added to the wells. After incubation for 2 h at 37 °C followed by washing with TBS/Tween, each well was filled with 200 ml of Block Ace solution (100 mg/ml) for 30 min at 37 °C. The anti-FDP-lysine monoclonal antibody (mAb5F6) was then added to the wells at 100 μl/well of 10 mg/ml solution for 3 h at 37 °C or overnight at 4 °C. After three washes with TBS/Tween, 100 μl/well peroxidase-conjugated anti-mouse IgG antiserum (1:4000) was added and incubated for 1 h at 37 °C. After washing, 100 μl of 0.05 M citrate buffer (pH 5.0) containing 0.4 mg/ml *o*-phenylenediamine and 0.003% H_2O_2 was added and incubated for several minutes at room temperature. The reaction was terminated by adding 2 M sulfuric acid, and the absorbance at 490 nm was read on a micro-ELISA plate reader.

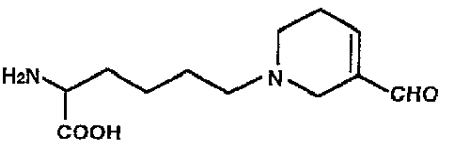


FIG. 1. **Chemical structures of FDP-lysine.**

SDS-Polyacrylamide Gel Electrophoresis—SDS-PAGE was performed according to Laemmli (13). The protein was stained with Coomassie Blue.

Immunoblot Analysis—A gel was transblotted onto a nitrocellulose membrane, incubated with Block Ace (40 mg/ml) for blocking, washed, and treated with mAb5F6. This procedure was followed by the addition of horseradish peroxidase conjugated to a goat anti-mouse IgG $F(ab')_2$ fragment and ECL reagents (Amersham Biosciences). The bands were visualized by exposure of the membranes to autoradiography film.

RESULTS

Inactivation of GAPDH by Acrolein-modified BSA—Based on the fact that the major acrolein-lysine adduct FDP-lysine (Fig. 1) retains an electrophilic α,β-unsaturated aldehyde moiety, we hypothesized that this adduct might react with nucleophiles such as the sulfhydryl groups of proteins via the Michael addition reaction. Hence, we examined the potential reactivity of the acrolein-modified proteins toward sulfhydryl enzymes using GAPDH as a convenient model protein that contains four sulfhydryl groups per subunit and is known to be highly sensitive to inactivation by α,β-unsaturated aldehydes *in vitro* (12). As shown in Fig. 2*A*, when GAPDH (0.1 mg/ml) was incubated with native BSA or acrolein-modified BSA (0–0.1 mg/ml) in 0.1 M sodium phosphate buffer (pH 7.2) for 24 h at 37 °C, the acrolein-modified BSA showed a significant inhibitory effect on the GAPDH activity. The concentration of acrolein-modified BSA up to 0.01 mg/ml had no detectable effect, whereas treatment with high concentrations (> 0.02 mg/ml) of the modified protein resulted in a significant reduction of the GAPDH activity; the enzyme activity decreased to 65% of the control values of the untreated GAPDH as the concentration of acrolein-modified BSA was increased to 0.1 mg/ml. In accordance with the loss of enzyme activity, the amount of cysteine (sulfhydryl groups) decreased to 50% of the initial value as the concentration of the acrolein-modified BSA was varied from 0 to 0.1 mg/ml (Fig. 2*B*). To examine the covalent binding of the acrolein-modified BSA with GAPDH, the incubation mixtures of the acrolein-modified BSA treated with GAPDH were analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 2*C*, the acrolein-modified BSA migrated as a broad protein band with the molecular mass of ~70 kDa, whereas the acrolein-modified protein was converted to a higher molecular mass protein species with >200 kDa, strongly suggesting that the acrolein-modified protein was covalently bound to GAPDH. These data suggest that acrolein modification of the protein results in the generation of an electrophilic adduct that possesses reactivity toward the thiol protein. This proposition was supported by the observation that pretreatment of the acrolein-modified BSA with GSH resulted in a decrease in the inhibitory effect on the GAPDH activity (Fig. 2*D*).

Covalent Binding of FDP-lysine to GAPDH—To examine whether FDP-lysine is involved in the inactivation of GAPDH upon incubation with acrolein-modified BSA, the enzyme was treated with various concentrations (0–1 mM) of N^{α}-acetyl lysine or N^{α}-acetyl-FDP-lysine in 0.1 M sodium phosphate buffer

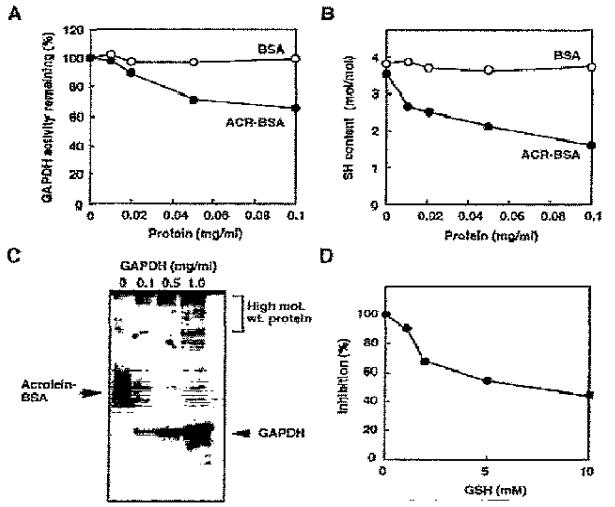


FIG. 2. **Inactivation of GAPDH by acrolein-modified protein.** *A*, changes in the enzyme activity of GAPDH upon incubation with acrolein-modified BSA. Symbols: ○, native BSA; ●, acrolein-modified BSA. *B*, loss of sulfhydryl contents in GAPDH upon incubation with acrolein-modified BSA. Symbols: ○, native BSA; ●, acrolein-modified BSA. *C*, formation of higher molecular mass protein species upon the incubation of GAPDH with acrolein-modified BSA. *D*, inhibitory effect of GSH on GAPDH inactivation by acrolein-modified BSA. The acrolein-modified BSA was prepared upon the incubation of BSA (1.0 mg/ml) with 10 mM acrolein in 50 mM sodium phosphate buffer (pH 7.2) at 37 °C for 24 h. The reaction mixture was then applied to a PD-10 column (Sephadex G-25) equilibrated with 0.1 M sodium phosphate buffer (pH 7.2) to separate the acrolein-modified protein from the free acrolein. In *panels A* and *B*, GAPDH (0.1 mg/ml) was incubated with native BSA or acrolein-modified BSA (0–0.1 mg/ml) in 0.1 M sodium phosphate buffer (pH 7.2) for 24 h at 37 °C. In *panel C*, acrolein-modified BSA (1.0 mg/ml) was incubated with GAPDH (0–1.0 mg/ml) in 0.1 M sodium phosphate buffer (pH 7.2) for 24 h at 37 °C. In *panel D*, acrolein-modified BSA (1.0 mg/ml) was treated with GSH (0–10 mM) in 0.1 M sodium phosphate buffer (pH 7.2) for 24 h prior to the incubation with GAPDH.

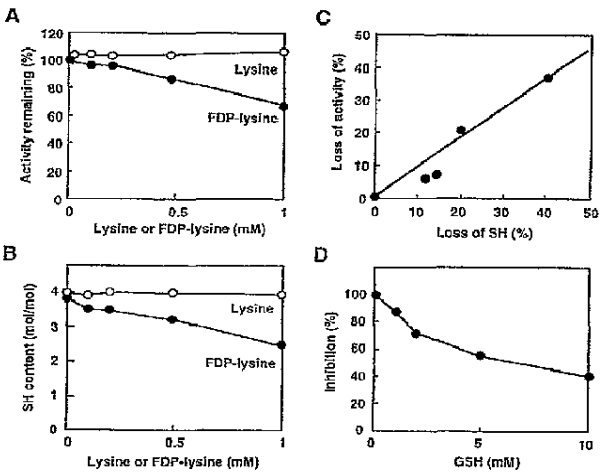


FIG. 3. **Inactivation of GAPDH by FDP-lysine.** *A*, changes in the enzyme activity of GAPDH upon incubation with FDP-lysine. Symbols: ○, N^{α}-acetyl lysine; ●, N^{α}-acetyl-FDP-lysine. *B*, loss of sulfhydryl groups in GAPDH upon incubation with FDP-lysine. Symbols: ○, N^{α}-acetyl lysine; ●, N^{α}-acetyl-FDP-lysine. *C*, stoichiometry of the loss of sulfhydryl (*SH*) groups and the loss of enzyme activity in GAPDH. *D*, inhibitory effect of GSH on GAPDH inactivation by FDP-lysine. In *panels A* and *B*, GAPDH (0.1 mg/ml) was incubated with various concentrations (0–1 mM) of N^{α}-acetyl lysine or N^{α}-acetyl-FDP-lysine in 0.1 M sodium phosphate buffer (pH 7.2) for 4 h at 37 °C. In *panel D*, N^{α}-acetyl-FDP-lysine (1 mM) was treated with GSH (0–10 mM) in 0.1 M sodium phosphate buffer (pH 7.2) for 24 h prior to the incubation with GAPDH.

(pH 7.2) for 4 h at 37 °C. As shown in Fig. 3*A*, the GAPDH activity decreased to 65% of the initial value as the concentration of the FDP-lysine derivative was varied from 0 to 1 mM. The loss of enzyme activity was associated with the decrease in the amount of the cysteine residues (sulfhydryl groups) (Fig. 3*B*). As shown in Fig. 3*C*, there is a linear correlation between the loss of catalytic activity and the loss of sulfhydryl groups from GAPDH. In addition, the inhibitory effect of FDP-lysine on the enzyme activity was significantly retarded by the addition of GSH (Fig. 3*D*).

To further examine the mechanism of inactivation of GAPDH caused by N^{α}-acetyl-FDP-lysine, the native and FDP-lysine-treated GAPDHs were analyzed by MALDI-TOF MS. As shown in Fig. 4, the analysis of native GAPDH revealed a peak with *m/z* 35,699. When GAPDH was incubated with 5 mM N^{α}-acetyl-FDP-lysine in 0.1 M sodium phosphate buffer (pH 7.2) for 24 h at 37 °C, some unmodified GAPDH subunits were observed (*m/z* 35,749) as well as the peaks (*m/z* 35,993, 36,263, and 36,539) corresponding to the addition of from one to three molecules of the FDP-lysine derivative per subunit. Sequential peaks in the spectrum of the FDP-lysine/GAPDH all differed in molecular mass by ~270–290 Da, values which were close to the molecular mass of N^{α}-acetyl-FDP-lysine (282 Da). This increase in the molecular mass provides strong evidence that the reaction between FDP-lysine and protein nucleophiles, such as thiols, occurred via the Michael addition reaction.

Thiolation of FDP-lysine—We then investigated the products formed by the reaction of the FDP-lysine with thiol compounds

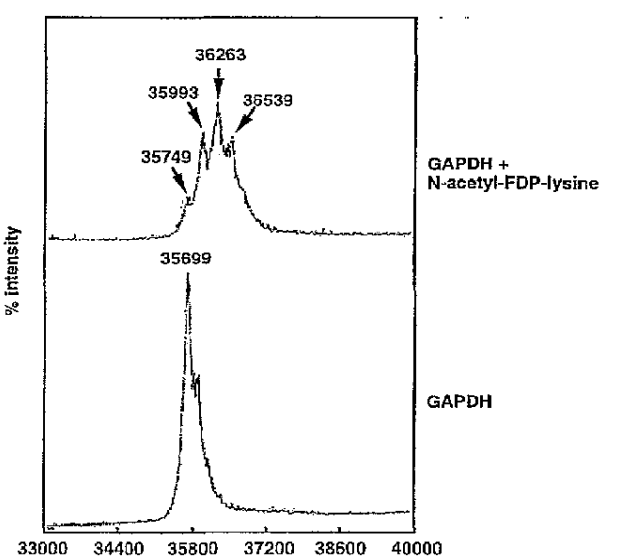


FIG. 4. **MALDI-TOF MS analysis of native (*bottom*) and FDP-lysine-treated GAPDH (*top*).** The FDP-lysine-treated GAPDH was prepared upon the incubation of GAPDH (0.1 mg/ml) with 5 mM N^{α}-acetyl-FDP-lysine in 0.1 M sodium phosphate buffer (pH 7.2) for 24 h at 37 °C.

such as N^{α}-acetylcysteine and GSH. As shown in Fig. 5, when 10 mM N^{α}-acetyl-FDP-lysine was incubated with 10 mM N^{α}-acetylcysteine (*panel A*) or 10 mM GSH (*panel B*) in 0.1 M sodium phosphate buffer (pH 7.2), the consumption of both the FDP-lysine and the thiol compounds was immediately observed within 1 h, suggesting the covalent binding of the FDP-lysine with these thiols. To detect an FDP-lysine-thiol conjugate, N^{α}-

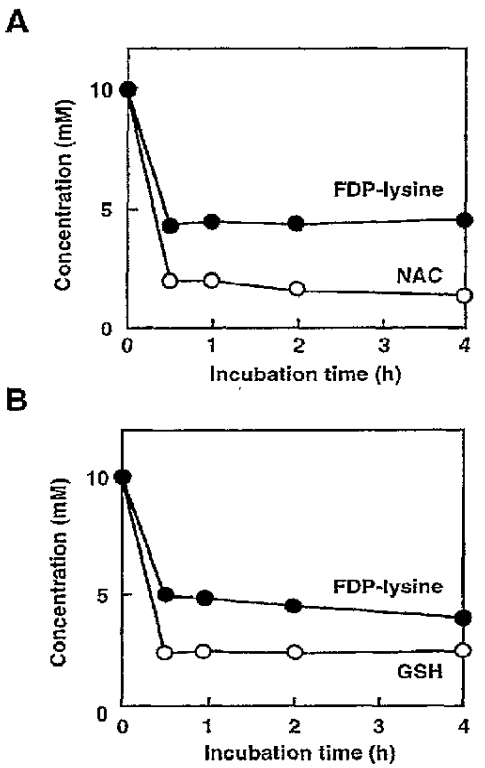


FIG. 5. **Reaction of FDP-lysine with thiol compounds.** N^{α}-Acetyl-FDP-lysine (10 mM) was incubated with an equimolar concentration of N^{α}-acetylcysteine (*A*) or GSH (*B*) in 0.1 M sodium phosphate buffer (pH 7.2). Symbols: ○, thiol compounds; ●, N^{α}-acetyl-FDP-lysine. The consumption of N^{α}-acetyl-FDP-lysine and thiol compounds was examined by reverse-phase HPLC using a Develosil ODS-HG-5 column (4.6 × 250 mm) (Nomura Chemicals) equilibrated in a solution of 5% methanol in 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min. The elution profiles were monitored by absorbance at 227 nm.

acetyl-FDP-lysine was incubated with an equimolar concentration of GSH in 0.1 M sodium phosphate buffer (pH 7.2) for 24 h, and the products were analyzed by LC-MS. As shown in Fig. 6*A*, the reaction of the FDP-lysine derivative with GSH indeed provided the new products (products *a* and *b*), which gave a pseudomolecular ion peak $(M+H)^+$ at *m/z* 589 (Fig. 6*B*) corresponding to the expected GSH conjugate with N^{α}-acetyl-FDP-lysine. Among other possibilities, the products *a* and *b* may represent the isomeric forms of the GSH-FDP-lysine conjugate. The GSH conjugates of FDP-lysine were relatively unstable, and we were not successful in stably isolating the conjugates. The adductions may be reversed in aqueous buffer, which leads to the reversible binding of GSH to FDP-lysine. The involvement of a Michael-type addition of a sulfhydryl group to the ethylenic bond of FDP moiety was also suggested by the observation that the UV light absorption spectrum of the N^{α}-acetyl-FDP-lysine showed a maximum at 227 nm, whereas the spectrum of the N^{α}-acetyl-FDP-lysine adducts of *N*-acetylcysteine and GSH exhibited a maximum at 204 nm.

It is notable that the GSH-FDP-lysine conjugate has a free aldehyde group that would be detectable by reaction with a carbonyl reagent. To examine whether the conjugate indeed possesses a carbonyl function, we exposed the reaction mixture of N^{α}-acetyl-FDP-lysine and GSH to an excess carbonyl reagent, 2,4-dinitrophenylhydrazine (DNPH), and the products were analyzed by reverse-phase HPLC. As shown in Fig. 6*C*, the products corresponding to the conjugates reacted with the carbonyl reagent and provided the new products, *c* and *d*. The LC-MS analysis of the products gave the same pseudomolecular ion peak $(M+H)^+$ at *m/z* 770 (Fig. 6*D*), which corresponded to the expected dinitrophenylhydrazone derivative of the GSH-FDP-lysine conjugate. These data also support our proposition that FDP-lysine reacts with GSH to form a GSH conjugate via a Michael addition reaction (Fig. 6*E*).

Glutathiolation of Acrolein-modified Protein—We have shown previously that, upon *in vitro* incubation of low-density lipoproteins with acrolein, the lysine residues that disappeared are partially recovered by the FDP-lysine (7). Based on the results of Figs. 2–6, it is likely that the FDP-lysine residues generated in the acrolein-modified proteins may react with GSH to form a glutathiolated protein. Prior to investigation of the glutathiolation of the acrolein-modified proteins, we examined the covalent binding of acrolein to the protein (BSA) and the formation of the FDP-lysine by MALDI-TOF MS and amino acid analysis, respectively. As shown in Fig. 7*A*, the MALDI-TOF MS analysis of the native BSA and acrolein-treated BSA revealed the peaks of 66,541 and 74,097, respectively, indicating that a substantial amount of acrolein was incorporated into the protein. When BSA (1 mg/ml) was incubated with 1 mM acrolein in 0.1 M sodium phosphate buffer (pH 7.2) for 24 h at 37 °C, the molecular mass increase that was observed corresponds to ~135 acrolein molecules per protein molecule, based on the 56-Da molecular mass of acrolein (Fig. 7*B*). We then determined the amount of FDP-lysine in the acrolein-modified BSA by amino acid analysis. As shown in Fig. 7*C*, the incubation of BSA with acrolein resulted in the loss of up to 51 lysine residues per mol of BSA at 24 h. These losses were accompanied by the formation of the FDP-lysine. The concentration reached about 20 molecules of FDP-lysine per protein molecule after a 1-h incubation. This accounted for about 40% of the lysine residues that had disappeared. Because formation of one FDP-lysine involves the loss of one H_2O molecule, and the corresponding mass shift would be 94, ~25% of the molecular mass increase in the acrolein (10 mM)-modified BSA can be accounted for by the formation of the FDP-lysine.

To examine whether acrolein-modified proteins react with GSH, the acrolein-modified BSA was incubated with GSH and analyzed by MALDI-TOF MS. As shown in Fig. 7*A*, the GSH-treated BSA did not show any change in molecular weight (*m/z* 66,596), whereas the GSH-treated acrolein-modified BSA gave a molecular ion peak, the center of which corresponds to *m/z* 78,339 as $(M+H)^+$. Upon incubation of the acrolein-modified BSA (1 mg/ml) with GSH (10 mM) at 37 °C, a time-dependent incorporation of GSH into the protein was observed (Fig. 7*D*). The binding of GSH to the acrolein-modified BSA reached a plateau after 6 h of incubation. On the basis of the known masses of GSH (307 Da), ~14 molecules of GSH were incorporated into the protein at 6 h.

FDP-lysine Residues as the Target of Glutathiolation—We have previously raised a monoclonal antibody (mAb5F6) against the acrolein-modified keyhole limpet hemocyanin and showed that the antibody recognizes the FDP-lysine as the major epitope (11). In the present experiments, we examined the changes in the immunoreactivity of the GSH-treated acrolein-modified protein with mAb5F6. As shown in Fig. 8*A*, the acrolein-modified BSA showed a potent immunoreactivity with the antibody, whereas incubation of the acrolein-modified BSA (1 mg/ml) with 10 mM GSH resulted in a significant decrease in the antigenicity of the protein (although the antigenicity of the acrolein-modified BSA was scarcely affected by 5 mM GSH). These data suggested that GSH is directly bound to the FDP-lysine residues in the acrolein-modified protein, leading to decreased immunoreactivity with the

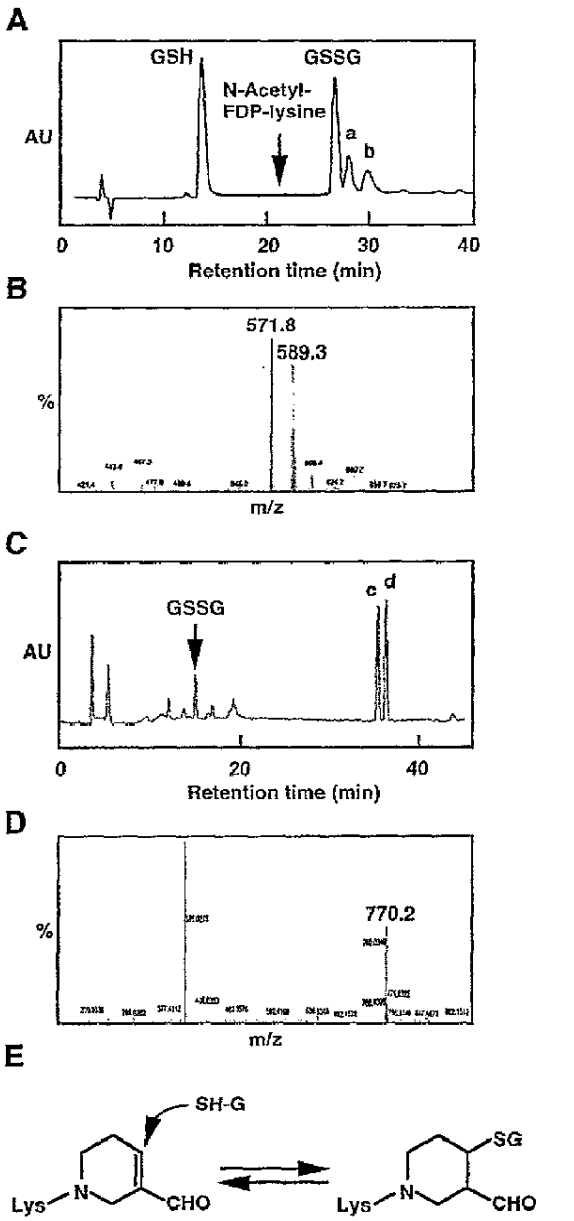


FIG. 6. **Formation of GSH-FDP-lysine conjugate.** *A*, HPLC profile of the reaction mixture of N^{α}-acetyl-FDP-lysine and GSH. N^{α}-Acetyl-FDP-lysine was incubated with an equimolar concentration of GSH in 0.1 M sodium phosphate buffer (pH 7.2) for 24 h, and the products were analyzed by reverse-phase HPLC using a Develosil ODS-HG-5 column (4.6 × 250 mm) (Nomura Chemicals) equilibrated in a solution of 5% methanol in 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min. The elution profiles were monitored by absorbance at 227 nm. *B*, mass chromatogram of *peak a*. *Peak b* gave a similar chromatogram as that of peak *a*. *C*, HPLC profile of the reaction mixture of N^{α}-acetyl-FDP-lysine and GSH after treatment with DNPH. An aliquot of the reaction mixture depicted in *panel A* was treated with an equal volume of 0.1% (w/v) DNPH in 2 N HCl and incubated for 1 h at room temperature. The mixture was analyzed with a reverse-phase HPLC using a Develosil ODS-MG-5 column (4.6 × 250 mm). The products were eluted with a linear gradient of 0.1% trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B) (time = 0, 100% solvent A; 40 min, 60% solvent A), at a flow rate of 0.8 ml/min. The elution profiles were monitored by absorbance at 195–650 nm. *D*, mass chromatogram of *peak c*. *Peak d* gave a similar chromatogram as that of peak *c*. *E*, schematic illustration of GSH binding to the FDP-lysine adduct. *AU*, absorbance unit.

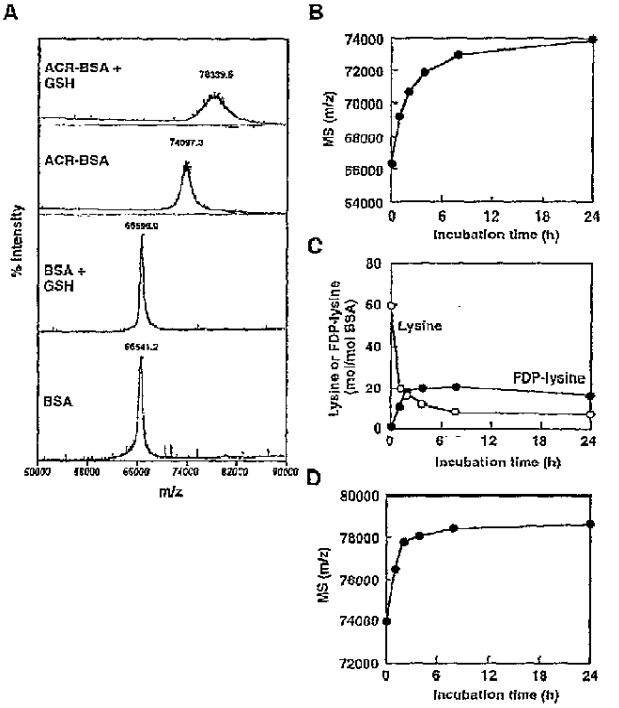
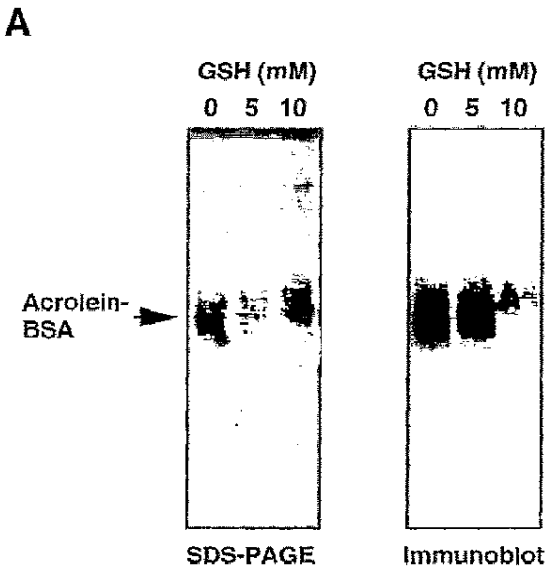


FIG. 7. **Covalent binding of GSH to the acrolein-modified BSA.** *A*, MALDI-TOF MS analysis of native BSA, GSH-treated BSA, acrolein-modified BSA, and GSH-treated acrolein-modified BSA. Acrolein-modified BSA was prepared upon the incubation of BSA (1 mg/ml) with 1 mM acrolein in 0.1 M sodium phosphate buffer (pH 7.2) for 24 h at 37 °C. The GSH-treated proteins were prepared upon the incubation of native BSA (1 mg/ml) or acrolein-modified BSA (1 mg/ml) with 10 mM GSH in 0.1 M sodium phosphate buffer (pH 7.2) at 37 °C. *B*, acrolein modification of BSA. The molecular weight increase in the acrolein-modified BSA was determined by MALDI-TOF MS. *C*, the loss of lysine residues and the concomitant formation of FDP-lysine residues in the acrolein-modified BSA. BSA (1.0 mg/ml) was incubated with 10 mM acrolein in 50 mM sodium phosphate buffer (pH 7.2) at 37 °C. *D*, binding of GSH to the acrolein-modified BSA. The molecular weight increase in the GSH-treated acrolein-modified BSA was determined by MALDI-TOF MS.

antibody. To further obtain evidence that the FDP-lysine residues generated in the acrolein-modified protein served as the binding-site of GSH, poly-L-lysine was coated on the immunoplate and sequentially incubated with acrolein and GSH, and changes in the immunoreactivity of the acrolein-modified poly-L-lysine with mAb5F6 after treatment with GSH were examined by ELISA. As shown in Fig. 8*B*, the immunoreactivity of the acrolein-modified poly-L-lysine with mAb5F6 was significantly decreased after treatment with GSH. These data strongly suggest that GSH primarily reacts with the FDP-lysine residues in the acrolein-modified proteins.

DISCUSSION

A growing body of evidence suggests that many of the effects of cellular dysfunction under oxidative stress are mediated by the products of the non-enzymatic reactions such as the peroxidative degradation of polyunsaturated fatty acids. Lipid peroxidation proceeds by a free radical chain reaction mechanism and yields the lipid hydroperoxides as the major initial reaction products. Subsequently, the decomposition of lipid hydroperoxides generates a number of breakdown products that display a wide variety of damaging actions. A number of reactive aldehydes derived from the lipid peroxidation have been implicated as causative agents in cytotoxic processes initiated by the exposure of biological systems to oxidizing agents (5). The previ-



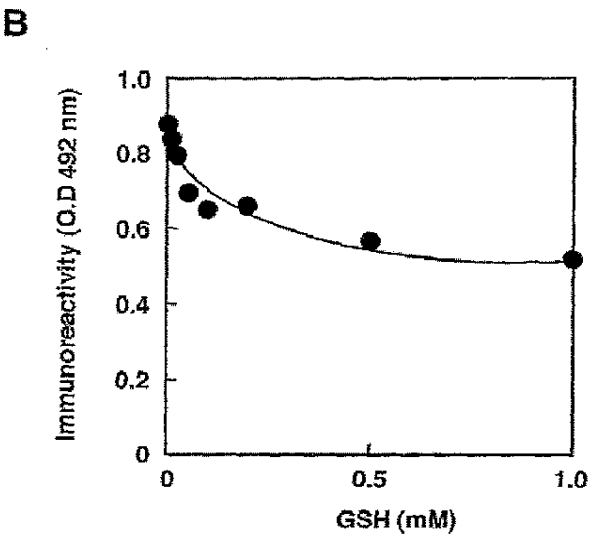


FIG. 8. **Changes in the immunoreactivity of GSH-treated acrolein-modified protein with mAb5F6.** *A*, SDS-PAGE (*left*) and immunoblot (*right*) analyzes of the GSH-treated acrolein-modified BSA. The acrolein-modified BSA (1 mg/ml) was incubated with GSH (0, 5, and 10 mM) in 0.1 M sodium phosphate buffer (pH 7.2) for 24 h at 37 °C. *B*, binding of GSH to the acrolein-modified poly-L-lysine. Poly-L-lysine coated on the immunoplate was incubated sequentially with acrolein and GSH, and the immunoreactivity of the GSH-treated acrolein-modified poly-L-lysine with mAb5F6 was examined using acrolein-trapping ELISA (see "*Experimental Procedures*").

ous observations that the metal-catalyzed oxidation of LDL is associated with the formation of the apoB-bound acrolein suggested that substantial amounts of acrolein might be generated during the peroxidation of polyunsaturated fatty acids in LDL (7). We have also observed that LDL peroxidation produces multiple products that react with 2,4-dinitrophenylhydrazine as monitored by reverse-phase HPLC, and one of the major products comigrates with the 2,4-dinitrophenylhydrazine derivative of authentic acrolein.[2] In addition, the same product

[2] A. Furuhata and K. Uchida, unpublished observations.

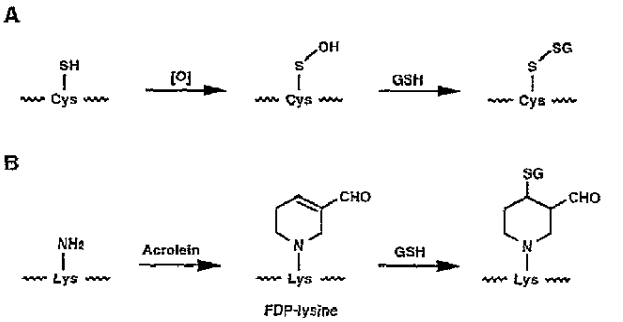


FIG. 9. **Schematic illustration of protein glutathiolation under oxidative stress.** *A*, glutathiolation of oxidized proteins. *B*, glutathiolation of acrolein-modified proteins.

has also been detected in the autoxidation of polyunsaturated fatty acids with an iron/ascorbate-mediated free radical generating system. These data strongly suggest that the peroxidation of polyunsaturated fatty acids represents a potential endogenous pathway for the production of acrolein. Although the mechanism of the formation of acrolein during lipid peroxidation has not yet been experimentally resolved, there may be no doubt that acrolein is a physiologically important aldehyde that could be ubiquitously generated under oxidative stress. Moreover, because acrolein is one of the most reactive and cytotoxic aldehydes, it could be a major causal factor that contributes to the development of tissue damage under oxidative stress.

Among all the α,β-unsaturated aldehydes, acrolein shows the greatest reactivity with nucleophiles such as proteins (5). We indeed observed that when BSA was treated with 2-alkenals and analyzed by MALDI-TOF MS, acrolein was the 2-alkenal most effectively incorporated into the protein.[2] Upon reaction with protein, acrolein selectively reacts with the side chains of the cysteine, histidine, and lysine residues. Of these, lysine generates the most stable product. The β-substituted propanals (R-NH-CH_2-CH_2-CHO) and Schiff's base cross-links (R-NH-CH_2-CH_2-CH = N-R) had been suggested as the predominant adduct; however, the major adduct formed upon the reaction of acrolein with protein was identified as a novel lysine product, FDP-lysine (Fig. 1), which requires the attachment of two acrolein molecules to one lysine side chain (7). The formation of a similar FDP-type adduct (dimethyl-FDP-lysine) has been reported in the lysine modification with the acrolein analogue crotonaldehyde (14). In addition, these FDP-type adducts have also been detected in the reaction of other 2-alkenals such as 2-pentenal and 2-hexenal with the lysine derivative (14), suggesting that the condensation reaction via the formation of the Michael addition-derived amine derivatives is characteristic of the reaction of 2-alkenals with primary amines. Because of the fact that the core structure of the FDP-lysine is resistant to the conventional acid hydrolysis of proteins even without reduction by pretreatment with sodium borohydride, the FDP adducts of acrolein and crotonaldehyde have been successfully detected not only in the acrolein-treated LDL but also in LDL exposed to metal-catalyzed oxidation (7). Furthermore, by use of a monoclonal antibody (mAb5F6) the detection of the FDP-lysine has so far been reported in plaque deposits of atherosclerotic lesions (11) and neurofibrillary tangles and plaque neuritic elements in Alzheimer's disease (15). These observations are in line with the accumulating body of literature supporting the role of oxidative stress in the pathogenesis of these disorders.

On the other hand, it is noteworthy that the treatment of BSA with acrolein resulted in the loss of about 50 molecules of

lysine residues but a gain of only 20 molecules of FDP-lysine moieties (Fig. 7*C*). The data strongly suggest that, upon reaction with lysine residues, acrolein could generate product(s) other than FDP-lysine. Candidate products may include propanal and the Schiff's base cross-link. The observations that the incubation of BSA with acrolein formed a faint protein band corresponding to the oligomer (dimer) of BSA (Figs. 2*C* and 3*A*) suggest the formation of the Schiff's base cross-link in the acrolein-modified protein. In addition, the formation of the pyridinium adducts has also been reported to be a dominant pathway for modification of the primary amine with 2-alkenals such as crotonaldehyde, 2-hexenal, and 2-octenal (14, 16–18), suggesting that a similar adduct might be formed upon the reaction of lysine residues with acrolein. Thus, the other 30 molecules of the lysine residues that had disappeared in the acrolein-modified BSA may be attributed to the formation of propanal, Schiff's base cross-link, and pyridinium-type adducts; however, their formation in the acrolein-modified protein has not yet been established.

The current study provides evidence that FDP-lysine is not a stable end product but a reactive intermediate that covalently binds to thiols. Based on the fact that FDP-lysine still retains the α,β-unsaturated aldehyde moiety, FDP-lysine was expected to have a potential reactivity with thiols. Indeed, FDP-lysine showed potent reactivities with the thiol compounds such as *N*-acetylcysteine and GSH (Fig. 5). The following lines of evidence suggest that the modification of FDP-lysine with thiols involves a Michael-type addition of the sulfhydryl group to the ethylenic bond of the FDP moiety (Fig. 6). (i) The UV light absorption spectrum of the N^{α}-acetyl-FDP-lysine adducts of *N*-acetylcysteine and GSH showed a maximum at 210 nm, whereas the spectrum of N^{α}-acetyl-FDP-lysine exhibited a maximum at 227 nm. (ii) The LC-MS analysis of the products in the GSH/N^{α}-acetyl-FDP-lysine incubation gave the M+H ion *m/z* 589, which would be expected from a Michael-type addition product composed of one molecule of N^{α}-acetyl-FDP-lysine and one molecule of GSH.

GSH, ubiquitously distributed in biological systems, is known to be an important molecule in defense against oxidative stress (19–21). The GSH-dependent processes are involved in the detoxification of α,β-unsaturated aldehydes such as acrolein, and the magnitude of the GSH loss reflects the magnitude of the decrease in resistance to the toxicity of reactive aldehydes (22, 23). It has been shown that the α,β-unsaturated aldehydes react with the sulfhydryl group of GSH via a Michael-type addition reaction, resulting in the formation of covalently bound aldehyde groups and the loss of sulfhydryls (5). This spontaneous conjunction of α,β-unsaturated aldehydes to GSH can be enhanced by at least two orders of magnitude by glutathione *S*-transferase (24). Therefore, it seems reasonable that the enzymes deplete the intracellular GSH. However, it is frequently observed that the loss of cellular GSH induced by reactive aldehydes is much greater than can be accounted for by this mechanism alone. This suggests the involvement of other mechanisms in the GSH depletion induced by reactive aldehydes. It may be important to consider an alternative mechanism of GSH depletion under oxidative stress in which reactive aldehydes generate the electrophilic adducts such as FDP-lysine, which could further exert depletion of GSH in the cells.

Protein thiolation is a reversible oxidative modification that involves the disulfide linkage of GSH or related endogenous low molecular weight thiols, *e.g.* cysteine, to select proteins *in vivo* (25, 26). Protein thiolation, in general, serves as an oxidative regulatory mechanism for certain enzymes and binding proteins with reactive cysteine residues. For example, proteins that have been shown to be negatively regulated by thiolation (glutathiolation) include the enzymes HIV protease (27) and aldose reductase (28) and the transcription factor Jun (29). The mechanism by which these stresses induce protein thiolation is poorly understood. Several mechanisms have been proposed for protein glutathiolation, including the following: (i) thiol-disulfide exchange between protein thiols and oxidized glutathione (30); (ii) oxidation of protein thiols by oxyradicals or H_2O_2 to form thiyl radicals or sulfenic acids and then to interact with GSH to produce mixed disulfide (31); (iii) nucleophilic attack of protein thiolate on *S*-nitrosoglutathione to produce a mixed disulfide (32–35); (iv) oxidation of GSH to form sulfenic acid and then interact with protein thiols to form mixed disulfides (33); and (v) nitrosation of protein thiols followed by interaction with GSH to form mixed disulfides (33, 34). These mechanisms for protein thiolation are represented by the formation of a disulfide linkage (protein *S*-thiolation). On the other hand, we found in this study that GSH was significantly incorporated into the acrolein-modified protein (Fig. 7). This and the observations that the incubation of acrolein-modified BSA or acrolein-modified poly-L-lysine with GSH resulted in a significant decrease in the immunoreactivity with the anti-FDP-lysine monoclonal antibody (Fig. 8) suggested that the FDP-lysine generated in the acrolein-modified protein apparently functioned as an electrophile and might be responsible for the thiolation of protein *in vivo*. Thus, this study established a novel mechanism for protein thiolation (protein *C*-thiolation) that utilizes the acrolein-lysine adduct (FDP-lysine) as a potential GSH-binding site (Fig. 9). Unlike protein *S*-thiolation, however, protein *C*-thiolation represents a modification of the FDP-lysine group and may not have any impact on protein functions.

In summary, we have characterized the reactivity of the acrolein-lysine adduct (FDP-lysine) toward free sulfhydryl groups and established a novel mechanism of protein thiolation in which the FDP-lysine adduct generated in the acrolein-modified protein undergoes the nucleophilic addition of thiols. These data suggest that the thiolation of protein-bound acrolein may be involved in the redox alteration under oxidative stress, whereby oxidative stress exerts an increased production of acrolein and its protein adducts that further potentiates oxidative stress via the depletion of GSH in the cells.

REFERENCES

1. Stadtman, E. R. (1992) *Science* **257**, 1220–1224
2. Stadtman, E. R. & Oliver, C. N. (1991) *J. Biol. Chem.* **266**, 2005–2008
3. Shacter, E. (2000) *Drug. Metab. Rev.* **32**, 307–326
4. Stadtman, E. R. & Levine, R. L. (2000) *Ann. N. Y. Acad. Sci.* **899**, 191–208
5. Esterbauer, H., Schaur, R. J. & Zollner, H. (1991) *Free Radic. Biol. Med.* **11**, 81–128
6. Uchida, K. (2000) *Free Radic. Biol. Med.* **28**, 1685–1696
7. Uchida, K., Kanematsu, M., Morimitsu, Y., Osawa, T., Noguchi, N. & Niki, E. (1998) *J. Biol. Chem.* **273**, 16058–16066
8. Kensler, C. J. & Battista, S. P. (1963) *N. Engl. J. Med.* **269**, 1161–1166
9. Izard, C. & Liberman, C. (1978) *Mutat. Res.* **47**, 115–138
10. Cohen, S. M., Garland, E. M., St. John, M., Okamura, T. & Smith, R. A. (1992) *Cancer Res.* **52**, 3577–3581
11. Uchida, K., Kanematsu, M., Sakai, K., Matsuda, M., Hattori, N., Mizuno, Y., Suzuki, D., Miyata, T., Noguchi, N., Niki, E. & Osawa, T. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 4882–4887
12. Uchida, K. & Stadtman, E. R. (1993) *J. Biol. Chem.* **268**, 6388–6393
13. Laemmli, U. K. (1970) *Nature* **227**, 680–685
14. Ichihashi, K., Osawa, T., Toyokuni, S. & Uchida, K. (2001) *J. Biol. Chem.* **276**, 23903–23913
15. Calingasan, N. Y., Uchida, K. & Gibson, G. E. (1999) *J. Neurochem.* **72**, 751–756
16. Alaiz, M. & Barragán, S. (1995) *Chem. Phys. Lipids* **75**, 43–49
17. Baker, A., Zidek, L., Wiesler, D., Chmelik, J., Pagel, M. & Novotny, M. V. (1998) *Chem. Res. Toxicol.* **11**, 730–740
18. Baker, A., Wiesler, D. & Novotny, M. V. (1999) *J. Am. Soc. Mass Spectrom.* **10**, 613–624
19. Sies, H. (1999) *Free Radic. Biol. Med.* **27**, 916–921
20. Meister, A. (1994) *Cancer Res.* **54**, Suppl. 7, 1969s–1975s
21. Rahman, I. & MacNee, W. (2000) *Free Radic. Biol. Med.* **28**, 1405–1420
22. Spitz, D. R., Malcolm, R. R. & Roberts, R. J. (1990) *Biochem. J.* **267**, 453–459

23. Spitz, D. R., Sullivan, S. J., Malcolm, R. R. & Roberts, R. J. (1991) *Free Radic. Biol. Med.* **11**, 415–423
24. Danielson, U. H., Esterbauer, H. & Mannervik, B. (1987) *Biochem. J.* **247**, 703–713
25. Thomas, J. A., Poland, B. & Honzatko, R. (1995) *Arch. Biochem. Biophys.* **319**, 1–9
26. Thomas, J. A., Chai, Y.-C. & Jung, C.-H. (1994) *Methods Enzymol.* **233**, 385–395
27. Davis, D. A., Newcomb, F. M., Starke, D. W., Ott, D. E., Mieyal, J. J. & Yarchoan, R. (1997) *J. Biol. Chem.* **272**, 25935–25940
28. Cappiello, M., Voltarelli, M., Cecconi, I., Vilardo, P. G., Dal Monte, M., Mariini, L., Del Corso, A., Wilson, D. K., Quiocho, F. A., Petrash, J. M. & Mura U. (1996) *J. Biol. Chem.* **271**, 33539–33544
29. Klatt, P., Molina, E. P. & Lamas, S. (1999) *J. Biol. Chem.* **274**, 15857–15864
30. Gilbert, H. F. (1990) *Adv. Enzymol. Relat. Areas Mol. Biol.* **63**, 69–172
31. Park, E.-M. & Thomas, J. A. (1988) *Biochim. Biophys. Acta* **964**, 151–160
32. Padget, C. M. & Whorton, A. R. (1998) *Arch. Biochem. Biophys.* **358**, 232–242
33. Ji, Y., Akerboom, T. P. M., Sies, H. & Thomas, J. A. (1999) *Arch. Biochem. Biophys.* **362**, 67–78
34. Mohr, S., Hallak, H., de Boitte, A., Lapetina, E. G. & Brüne, B. (1999) *J. Biol. Chem.* **274**, 9427–9430
35. Percival, M. D., Ouellet, M., Campagnolo, C., Claveau, D. & Li, C. (1999) *Biochemistry* **38**, 13574–13583
36. Hissin, P. J. & Hilf, R. (1976) *Anal. Biochem.* **74**, 214–216